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EFFECT OF THE THIOL-OXIDIZING AGENT, DIAMIDE, ON CEREBRAL CORTICAL Na^+-K^+ ATPase

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TECHNICAL REVIEW AND APPROVAL

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The experiments reported herein were conducted according to the principles set forth in the current edition of the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

This technical report has been reviewed by the NMRI scientific and public affairs staff and is approved for publication. It is releasable to the National Technical Information Service where it will be available to the general public, including foreign nations.

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This study documents the action of the thiol-oxidizing agent, diamide, on the Na^+,-K^+, ATPase, and compares it with other sulfhydryl inhibitors. Diamide inhibits the activity of in vitro cerebral cortical and whole brain synaptosomal Na^+,-K^+, ATPase. Complete inhibition occurs at 0.1 M diamide, with a progressive increase in enzyme activity occurring sigmoidally to 100% activity at 1 μM diamide. The diamide-induced inhibition of the Na^+,-K^+, ATPase activity is not reversed by washing with diamide-free media. However, partial reversal of inhibition is possible when the diamide-treated enzyme is incubated with the thiol-reducing agents dithiothreitol or cysteine. Partial protection against diamide-induced sulfhydryl oxidation was obtained in the presence of Na^+, and ATP, whereas, addition of Mg^{2+}, and K^+, decreases the level of protection. Therefore, the reactivity of diamide on the sulfhydryl groups of the catalytic subunit is dependent upon the conformational state of the enzyme.

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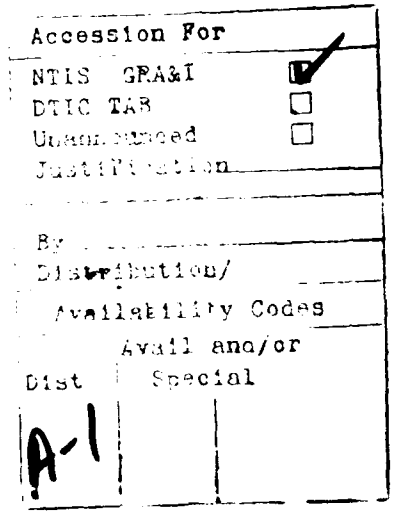
The experiments reported herein were conducted according to the principles set forth in the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources. National Research Council, DHHS, Pub. No. (NIH) 86-23.



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INTRODUCTION

It has been well documented that the nervous system is particularly susceptible to oxidative damage. For example, exposure to 100% oxygen at pressures above 2 ATA produces seizures in man and experimental animals. Divers breathing 100% oxygen at depths greater than 33 feet are therefore confronted with the ominous prospect of such convulsions and possible drowning. For this reason, it is essential to understand the mechanisms by which biological oxidations induce nervous system dysfunction.

Diamide is a drug that mimics various aspects of hyperbaric oxygen and thus may serve as a valuable tool for exploring the mechanism(s) by which oxygen alters normal CNS function.

Diamide, $(CH_3)_2NCON{=}NCON(CH_3)_2$, is a thiol-oxidizing agent (1,2) whose biological activity was first shown to be a potent oxidizer of intracellular glutathione (GSH) (2,3,4). Diamide has since been shown to inhibit Ca^{2+} uptake by <u>in vitro</u> rat liver (5) and rabbit whole brain mitochondria (6), to inhibit ouabain insensitive Mg^{2+} and Ca^{2+} ATPases from red blood cells (7), and either stimulate (8) or inhibit (9) mitochondrial proton conductivity and H^+-ATPase activity. Although it is unclear whether diamide stimulates or inhibits mitochondrial proton conductance and ATPase activity, it has been shown by both groups (8,9) that diamide affects mitochondrial H^+-ATPase enzyme activity primarily by the oxidation of vicinal (i.e., juxtaposed) dithiols. Access to these dithiol groups are dependent on the conformational state of the enzyme. For example, diamide inhibition of RBC Mg^{2+} ATPase is reported to be essentially 100% at 2 mM diamide whereas in the presence of Ca^{2+}, up to 4 mM diamide does not suppress activity beyond 55% (7,10). In this instance, Ca^{2+} is attributed with inducing a conformational change in the enzyme

structure, therefore masking critical SH groups from oxidation and subsequent inactivation (10).

The Na^+-K^+ ATPase (EC 3.6.3.1) is a sulfhydryl-rich enzyme, whose activity and conformational state is influenced by ligand composition, temperature, and/or pH of the incubation media (11-15). The susceptibility of this enzyme to thiol-oxidation is also dependent upon its conformational state and the nature of the sulfhydryl-oxidizing agent (16-19). It was of interest, therefore, to document the action of diamide on the Na^+-K^+ ATPase, and compare its effects with other sulfhydryl inhibitors.

MATERIALS AND METHODS

The assays were performed with sodium-potassium activated adenosine 5'-triphosphatase (ATPase; Sigma Chemical Co.) that was extracted with sodium iodide from hog cerebral cortex (20), or with ATPase prepared in our laboratory from rabbit whole brain synaptosomes that were freeze-fractured and further homogenized. Because background activity in this fragmented membrane preparation was low, no further purification was required. The commercially obtained ATPase was prepared by suspending approximately 5.8 units ATPase per 16.2 mg protein (1 unit = 1 μmole P_i/min) in 40 ml of ATPase preparation media (50 mM Tris and 1 mM EDTA at pH 7.65).

The experiments followed two protocols. The first method entailed the addition of 0.2 ml ATPase solution to centrifuge tubes containing 1.1 ml incubation media (100 mM NaCl, 25 mM KCl, 12 mM $MgCl_2$, 1mM Na_2 EDTA, and 50 mM Tris at pH 7.65) (diamide-free) or to 1.1 ml incubation media plus diamide. The tubes were preincubated for 10 min at 37 °C and then 0.2 ml of adenosine 5'-triphosphate (ATP; Sigma Chemical Co.) in the appropriate concentration (see below) was added to each tube to initiate the reaction. The final volume

of 1.5 ml produced a solution containing approximately 0.05 mg protein/ml. Each tube was incubated for 20 min and then the reaction was stopped with 0.5 ml of ice-cold 1.2 M perchloric acid containing 27 mM silicotungstic acid. Each tube was placed on ice and inorganic phosphate was determined by the assay procedure described by Post and Sen (21).

The second protocol entailed the preincubation of 5 ml of ATPase solution at 37 °C for 10 min with or without diamide. The ATPase solutions were then pelleted by centrifugation at 99,000 x g at R_{max} in a swinging bucket rotor (Beckman SW50) for 25 min. The pellet was rinsed once with ATPase preparation media and resuspended in 5 ml of diamide-free ATPase preparation media. The ATPase was again centrifuged, rinsed, and resuspended in 5 ml of diamide-free ATPase media. These manipulations were performed to remove any residual diamide from the preparation media. The ATPase assay was then performed on 0.2 ml aliquot of the pretreated ATPase.

The ATP concentration is expressed as $MgATP^{-2}$ (its active form). The technique using excess $MgCl_2$ was applied because $MgATP^{-2}$ concentration has been shown to vary when the concentration of metal ion and nucleotide are adjusted in a constant ratio (22). For all concentrations of ATP, an excess of Mg^{2+} between 5 and 10 mM existed, producing 85-90% $MgATP^{-2}$. ATPase activity was assayed against a 1 mM ouabain-containing blank in order to subtract out phosphate originating from sources other than the Na^+-K^+ ATPase.

RESULTS

The Effect of Diamide on Na^+-K^+ Activated ATPase Activity

Preliminary data indicated that the porcine and rabbit brain ATPases responded to diamide in a qualitatively similar manner. Therefore, the porcine ATPase was used to perform the majority of these studies. To

determine the effects of diamide on the sodium-potassium activated ATPase, the enzyme was incubated in the presence of 3.6 mM ATP and varying amounts of diamide. In the range of 1 μM to 0.1 M diamide, ATPase activity varied from 0 to 100% inhibition, respectively (Fig. 1). The activity changed sigmoidally with 50% activity occurring at approximately 2.5×10^{-4} M. The dose-response curve remained the same for both protocols.

Reversibility by Thiol-Reducing Agents

Washing the diamide-exposed enzyme with ATPase preparation media did not restore the enzyme activity. However, activity was restored if a thiol-reducing compound antagonized the oxidizing effects of diamide. This was shown by preincubating the enzyme with diamide for 10 min, then pelleting, washing, and subsequently resuspending in dithiothreitol (DTT) containing media. The enzyme-DTT solution was preincubated for 20 min, then the wash procedure was repeated. The enzyme was incubated in DTT-free media with 3.6 mM ATP as described in the Methods section. As the proportion of DTT to diamide was increased from 1:10 to 25:1, enzyme activity increased (Table 1), indicating a progressive (but not complete) reversal of the oxidizing effect of diamide. A similar effect was noted when diamide containing solutions were incubated in the presence of cysteine (Table 2).

When the enzyme was incubated in the presence of 1.25×10^{-3} M N-ethylmaleimide (NEM) and then followed with DTT treatment as described above, it was observed that DTT to NEM ratios as high as 80:1 produced no reversal of NEM inhibition on the enzyme.

Kinetic Analysis of Diamide Action

To enhance the understanding of the Na^+-K^+ ATPase-diamide interaction, kinetic analysis was applied. A Lineweaver-Burk plot of the enzyme-diamide interaction indicated that 1×10^{-4} M diamide produced a decrease in Vmax from

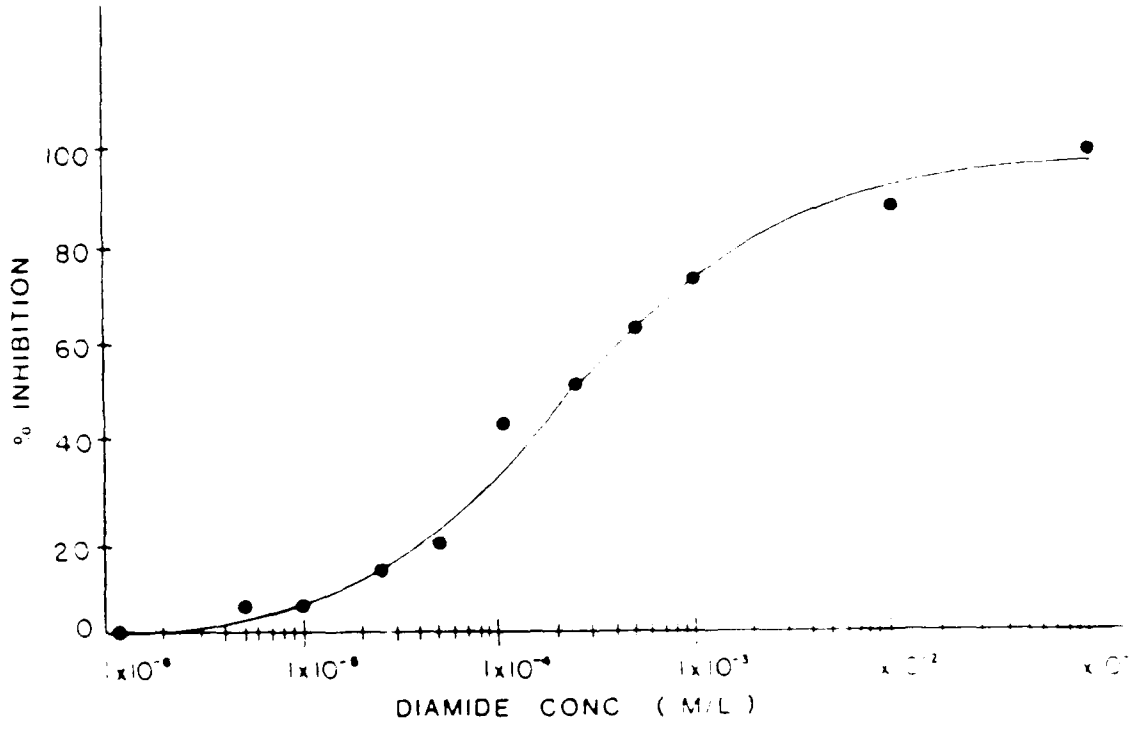

Figure 1. Enzyme activity response curve demonstrating the percent inhibition of ATPase activity induced by varying concentrations of diamide. In the range of 1 micromolar to 0.1 molar diamide, ATPase activity varied from 0 to 100% inhibition, respectively. Each point on the curve represents the average value for 8 experiments.

a control value of 47.9 to 28.8 mMole P_i/hr/mg protein, while Km remained unchanged at 6.49×10^{-4} M.

The Effect of Changes in Ionic Composition on Diamide Action

The degree of inhibition of the Na^+-K^+ ATPase activity by NEM has been related to the ionic composition of the incubation media (11,12,17,18,23). Magnesium has been shown to potentiate NEM inhibition, whereas Na^+ and ATP provide protection against NEM (12). Similar ionic manipulations were performed using 1.25×10^{-3} diamide in standard incubation media (i.e., containing Na^+ + K^+ + Mg^{2+} + ATP) and in K^+ and Mg^{2+} free incubation media. The K^+-Mg^{2+} free media provided protection against diamide when compared to the activities obtained from the K+ and Mg^{2+}-containing solutions (Table 3). Under identical conditions, 1.25×10^{-3} M NEM produced results similar to those found with diamide (Table 3).

Effect of Diamide on the Membrane Lipid Matrix

The ATPase was exposed for 15 min to either 10 mM diamide or to the lipid peroxidizing agent lipoxidase (55,350 units). This amount of lipoxidase produced no significant change in enzyme activity, but did generate 0.0663 ± 0.08 nMoles of malondialdehyde (an end product of lipid peroxidation). With 10 mM diamide, evidence of lipid peroxidation could not be detected. The effect of diamide therefore appears to be directed towards the protein moiety of the enzyme and not the lipid matrix.

DISCUSSION

Diamide (diethyl azodicarboxylate) has been shown to function as a strong electron acceptor, with a powerful tendency to extract hydrogen atoms from various hydrogen donors. It has been determined that this compound undergoes hydrogenation to diethyl hydrazodicarboxylate plus yielding a disulfide from the sulfhydryl donor (1,3,4).

In this study, diamide directly inhibited the activity of in vitro porcine cerebral cortical and whole brain synaptosomal Na^+-K^+ activated ATPase. Complete inhibition occurred at 0.1 M diamide with a progressive increase in enzyme activity occurring sigmoidally to 100% activity at 1 μM diamide. The diamide-induced inhibition of the Na^+-K^+ ATPase activity could not be reversed by washing with diamide-free preparation media. However, partial reversal of inhibition was possible when the diamide treated enzyme was incubated with the thiol-reducing agents, DTT or cysteine. The DTT or cysteine induced reversal of inhibition was corroborative of diamide's action as a sulfhydryl oxidizing agent.

The degree and rate of disulfide formation is dependent upon the proximity of the SH groups within the molecule (i.e., the degree of separation of the SH groups) as well as the nature, charge and proximity of neighboring side groups (24,25). Because of the susceptibility of the ATPase to thiol oxidation by diamide, it is likely that the SH groups are sufficiently contiguous within the enzyme to form internal disulfides. The nature and location of these SH groups can also account for the difference in vulnerability of the enzyme for oxidation by DTNB and alkylation by NEM. This concept is supported by findings that diamide induces internal disulfides in coupling factor B (F_B) from beef heart mitochondria (8,9,26,27). Factor B is a water soluble component of the H^+-ATPase which contains two essential contiguous (vicinal) dithiols (26,28).

Modulation of enzyme activity in response to sulfhydryl oxidation with diamide was obtained in an incubation media of differing ligand composition. Protection against sulfhydryl oxidation was obtained in the presence of Na^+ and ATP; addition of Mg^{2+} and K^+ to the incubation media decreased the level of protection (i.e., decreased enzyme activity). Therefore, the reactivity of

diamide on the sulfhydryl groups of the catalytic subunit was dependent upon the conformational state of the enzyme and is similar to that observed with NEM (11,12,17,18,19).

The inhibition of the Na^+-K^+ ATPase activity by NEM and DTNB has been reported to obey second-order kinetics, with inhibition resulting from the reaction of one molecule of inhibitor with at least one of several "vital" groups on the ATPase (19,23). Kinetic analysis of the diamide-ATPase interaction is suggestive of a similar mode of action. The supposition of multiple "vital" groups is strengthened by the observation that conformational alteration of the enzyme does not provide complete protection.

The type of lipid matrix surrounding the ATPase has been shown to influence the activity of this enzyme (29), therefore, the effect of diamide on lipid peroxidation was observed. Diamide did not produce a detectable amount of oxidation on the lipid matrix of the membrane. Whether membrane fluidity was altered, however, is unknown. Thus, diamide appears to exert its principle effect on the sulfhydryl component of the protein through sulfhydryl oxidation, and the formation of internal disulfides at critical locations within the enzyme.

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TABLE 1. Reversibility of the diamide induced inhibition of Na-K ATPase activity by dithiothreitol (DTT)*

Mean ± S.E.
(n)

*Washing diamide exposed enzyme with preparation media would not restore enzyme activity. However, treatment (see text) of the diamide exposed enzyme with various concentrations of dithiothreitol produced a progressive recovery of activity.

†μPi/hr/mg protein

Diamide:DTT ratio	Diamide/DTT concentration	Control activity†	Post-treatment activity	% control activity
---	1×10^{-3} M diamide	25.68 ± 1.18 (4)	7.19 ± 0.95 (4)	27.68
---	1×10^{-2} M diamide	29.46 ± 1.69 (4)	3.68 ± 0.41 (4)	12.49
---	1×10^{-2} M DTT	28.75 ± 0.78 (4)	30.85 ± 1.36 (4)	107.30
10:1	1×10^{-2} M/1×10^{-3} M	33.78 ± 1.14 (4)	8.51 ± 1.21 (4)	25.19
1:1	1×10^{-2} M/1×10^{-2} M	33.33 ± 1.41 (4)	12.86 ± 0.89 (4)	38.58
1:10	1×10^{-3} M/1×10^{-2} M	26.62 ± 1.14 (4)	20.40 ± 1.38 (4)	76.63
1:25	1×10^{-3} M/2.5×10^{-2} M	28.46 ± 1.12 (4)	24.16 ± 1.01 (4)	84.89

TABLE 2. Reversibility of the diamide induced inhibition of Na-K ATPase activity by cysteine*

Mean ± S.E.
(n)

*Same conditions as cited for Table 1 except cysteine was utilized as the thiol reducing compound.

†μPi/hr/mg protein

Diamide:cysteine ratio	Diamide/cysteine concentration	Control activity†	Post-treatment activity	% control activity
---	1×10^{-4} M diamide	34.44 (12)	21.82 (12)	63.35
1:1	1×10^{-4} M/1×10^{-4} M	37.90 (4)	28.40 (4)	74.93
1:10	1×10^{-4} M/1×10^{-3} M	31.77 (5)	26.41 (5)	83.13
1:100	1×10^{-4} M/1×10^{-2} M	33.66 (3)	32.29 (3)	95.93

TABLE 3. Protection against diamide induced inhibition by a K^+-Mg^{++} free media*

Mean ± S.E.
(n)

*The degree of inhibition of Na^+-K^+ ATPase activity by thiol alkylation or oxidation is related to the ionic composition of the incubation media. For both diamide and N-ethylmaleimide induced inhibition of enzyme activity, removal of K^+ and Mg^{++} from the incubation media produced an increase in enzyme activity when compared to enzyme incubated in K^+ and Mg^{++} containing media.

+μP_i/hr/mg protein

**Paired 't' test applied for K^+ and Mg^+ free versus K^+ and Mg^{++} containing.

$P < 0.01$.

Inhibitor (1×10^{-3})	Activity control	Activity+ K & Mg free	Activity K & Mg containing
Diamide	11.71 ± 3.15 (8)	3.27 ± 1.07** (8)	0.868 ± 0.875** (8)
NEM	12.09 ± 1.23 (8)	5.93 ± 1.16** (8)	2.18 ± 1.09** (8)